

ACTION OF RENNIN ON κ -CASEIN, THE AMINO ACID COMPOSITIONS OF THE PARA- κ -CASEIN, AND GLYCOMACROPEPTIDE FRACTIONS

E. B. KALAN AND J. H. WOYCHIK

Eastern Regional Research Laboratory, Eastern Utilization Research and Development ~~Division~~
U.S.D.A., Philadelphia, Pennsylvania

ABSTRACT

The action of rennin on κ -casein obtained from the milk of an individual cow was investigated. Polyacrylamide-gel electrophoresis at pH 9.2 indicated the presence of two major components in reduced para- κ -casein. The amino acid compositions of κ -casein, para- κ -casein, and the glycomacropeptide were compared.

The action of rennin on casein of cow's milk has recently been reviewed by Lindqvist (14a, b) and by Alais and Jollès (2a, b, c). It has been shown by Nitschmann and co-workers (16, 21, 23) that the action of rennin consists of a primary enzymic phase in which the protective colloid of casein is destroyed, followed by a secondary phase in which a coagulum forms. A tertiary, nonspecific proteolytic phase has also been demonstrated (4). Alais (1) and later Nitschmann and Henzi (20) showed that the primary action of rennin results in the formation of a nondialyzable glycomacropeptide (GMP) soluble in 12% trichloroacetic acid (TCA). The primary reaction has been shown (29, 31) to be confined to the κ -casein fraction first described by Waugh and von Hippel (32).

The GMP consists of about 30% carbohydrate and 70% peptide material and about 0.4% phosphorus (9, 10), having a molecular weight of 6,000-8,000 (10, 22, 30). The carbohydrate portion is made up of galactose, galactosamine, and sialic acid (20, 22). The peptide portion has been reported (3, 8-10, 19) to consist of 11 amino acids, being low in, or devoid of, the sulfur amino acids, aromatic amino acids, and histidine and arginine. Jollès et al. (10) have also reported the presence of one residue per mole of an unknown amino acid. More recently, Delfour et al. (6) have reported the presence of one methionine residue in the glycomacropeptide derived from both cow and sheep κ -casein, which appears to be the N-terminal residue.

Analyses of para- κ -casein, the coagulum formed during the secondary phase of the action of rennin, have been limited to those of Jollès et al. (3). They have found that for the most

part the sum of the individual amino acid residues found in the GMP and para- κ -casein approximate the number found in κ -casein, whose amino acid composition was reported by Jollès et al. (11). κ -Casein and the GMP had the same C-terminal amino acids, valine, alanine, threonine, and serine, whereas para- κ -casein yields leucine and phenylalanine. Treatment of κ -casein with LiBH_4 (12) yielded para- κ -casein-like material with phenylalaninol as the C-terminal group, and glycopeptide. On the basis of this evidence, the action of rennin is presumed to be esterolytic, cleaving an ester link between the carboxyl of phenylalanine and a hydroxyl group of the GMP, which arises from the C-terminal portion of κ -casein. The most recent report (6) would seem to throw doubt on the esterolytic theory of rennin action.

In the past, most of the investigations of the action of rennin have utilized casein preparations from pooled milk. Recently, it has been shown that reduced κ -caseins exhibit a heterogeneity presumably related to genetic polymorphism (15, 18, 26, 34, 35). Two major components occur either singly or in pairs in the reduced κ -caseins of individual cows, usually accompanied by four to six minor components. Each of these components has the ability to stabilize α_{s1} -casein and can serve as a substrate for rennin (15, 36). These facts emphasize the desirability of utilizing preparations obtained from single, typed cows, containing a single major component. The present communication reports results of an investigation of the action of rennin on κ -casein obtained from a single, Type b cow (18). These initial studies report the amino acid compositions and the gel-electrophoresis behavior of κ -casein, para- κ -casein, and the GMP.

MATERIALS AND METHODS

κ -Casein was isolated from acid-precipitated whole casein obtained from a single, Type *b* (18) cow by the urea-sulfuric acid method of Zittle and Custer (27). The isolated κ -casein was further purified by precipitation from alcoholic solution (17).

κ -Casein (0.5%) was digested with crystalline rennin (Sigma Chemical Company, St. Louis¹) (1.0 μ g/ml) in pH 6.7 citrate buffer (0.01 M) containing 0.1 M sodium chloride. After 10 min digestion, the para- κ -casein was removed by filtration and washed three times with water and dialyzed overnight. An equal volume of 24% TCA was added to the filtrate and a slight turbidity developed which was removed by centrifugation. This material was presumed to be unreacted κ -casein or material arising from the proteolysis of either κ -casein or para- κ -casein as a result of the tertiary phase of the rennin reaction (4). This fraction was not examined further. The 12% TCA solution of GMP was then adjusted to pH 8.0 with 1 N sodium hydroxide and dialyzed. The para- κ -casein and GMP fractions were lyophilized

¹ It is not implied that the U. S. Department of Agriculture recommends products of companies mentioned to the possible exclusion of others in the same business.

after dialysis. Two separate GMP preparations were obtained. Each was analyzed with essentially the same results; therefore, results of only one preparation are reported.

κ -Casein, para- κ -casein, and GMP were analyzed for their complete amino acid compositions by the method of Piez and Morris (25), using the Phoenix Precision Instrument Company automatic analyzer, Model VG-6000-B.¹ Approximately 2.0 mg of samples were hydrolyzed with glass-distilled 6 N HCl in sealed, evacuated tubes. Hydrolyses were carried out at 110 C in a circulating-air laboratory oven, in triplicate for 24, 72, and 96 hr. Data reported in Table 1 were calculated by finding molar ratios based on eight different amino acids (Asp, Pro, Ala, Met, Leu, Phe, His, and Arg) for κ -casein and para- κ -casein. For the GMP, Asp, Lys, Leu, Ala, and Glu were used to calculate the molar ratios. The molar ratios so obtained were multiplied by whole number factors so that the ratios derived from each of the amino acids were raised to the same numerical level. Cystine was determined as cysteic acid following performic acid oxidation (27).

Polyacrylamide-gel electrophoresis in pH 9.2 Tris-4.5 M urea was carried out according to the method already reported (24). Electrophoresis at pH 4.0 was performed in an alumi-

TABLE 1
Amino acid composition ^a of κ -casein, para- κ -casein, and glycomacropeptide (residues)

Amino acid	κ -Casein		para- κ -Casein		Difference between κ and para- κ		GMP	
Aspartic acid	11.0	.29 ^d	8.2	.30 ^d	2.8	3 ^e	3.3	.26 ^d 3 ^e
Threonine ^b	12.1	5.2	6.9	7	7.4 7
Serine ^b	11.0	7.6	3.4	3	3.4 3
Glutamic acid	24.9	.30	17.9	.36	7.0	7	8.7	.64 9
Proline	17.5	.21	12.9	.27	4.6	5	5.3	.35 5
Glycine	2.6	.04	1.7	.03	0.9	1	.8	.05 1
Alanine	13.4	.16	9.7	.20	3.7	4	4.0	.33 4
½ Cystine ^c	1.9	.05	1.8	.04	0.1
Valine	10.2	.11	6.5	.12	3.7	4	3.9	.32 4
Methionine	1.9	.04	1.3	.02	0.6	1	.6	.04 1
Isoleucine	11.5	.11	7.4	.14	4.1	4	4.8	.39 5
Leucine	8.7	.10	8.0	.16	0.7	1	.6	.02 1
Tyrosine	8.8 ^b	8.8	.16	0.0
Phenylalanine	4.2	.05	4.1	.08	0.1	..	.2	.01 ..
Lysine	9.1	.11	7.0	.14	2.1	2	2.5	.18 2-3
Histidine	3.0	.05	2.9	.06	0.1
Arginine	5.0	.07	5.0	.10	0.0	..	.3	.02 ..
Total residues ^f	156.8		116.0		42			45-46

^a Values based on molar ratios (see text) obtained from triplicate analyses for each of three hydrolysis periods (24, 72, and 96 hr).

^b These values were estimated using linear regression analysis.

^c Determined as cysteic acid on performic acid-oxidized samples.

^d Measure of repeatability at the 95% confidence limit.

^e Nearest integer.

^f Exclusive of tryptophan.

num lactate buffer (13)—8 M urea, with ionic strength of 0.025. The protein patterns were developed with Amido Black.

RESULTS AND DISCUSSION

The κ -casein was attacked by rennin at the same rate as previously established with pooled κ -casein (36). After maximum formation of para- κ -casein and GMP (10 min), the fractions were isolated as described in the methods. The electrophoretic patterns of para- κ -casein and GMP were compared with the original κ -casein at pH 9.2; results are presented in Figure 1.

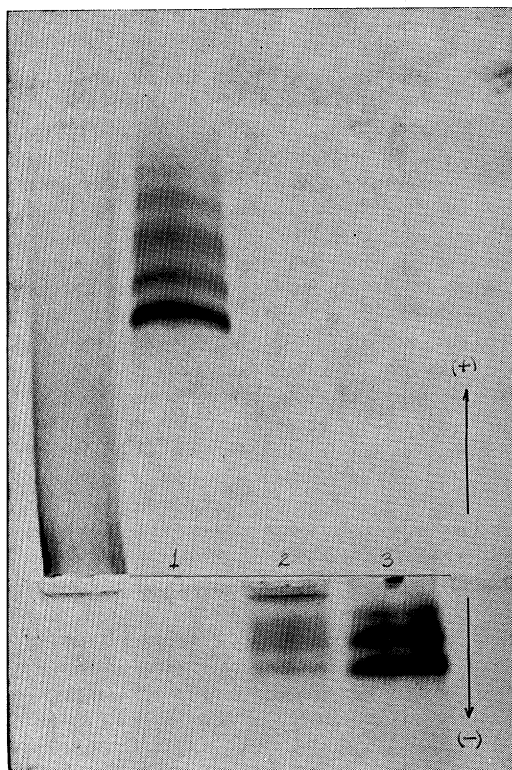


FIG. 1. Polyacrylamide-gel electrophoresis of κ -casein and para- κ -casein in Tris buffer, pH 9.2-4.5 M urea. Reduced κ -casein (1), para- κ -casein (2), and reduced para- κ -casein (3).

Pattern 1 is that of the original κ -casein, reduced with mercaptoethanol to show the major and the minor components. The para- κ -casein (Pattern 2) in the unreduced form showed limited solubility; however, a slight pattern was discernible. On the other hand, reduced para- κ -casein (Pattern 3) was freely soluble and appeared to migrate completely into the gel. In contrast to the whole κ -casein, the para- κ -casein migrated toward the cathode and con-

sisted of two major and one minor component. No migration toward the anode was observed, indicating the absence of unaltered κ -casein. No pattern was detected for the GMP fraction, which could perhaps be attributed to three factors: First, the highly acidic character of the macropeptide could have resulted in a rapid migration off the anodic portion of the gel, or secondly, the high solubility (of the GMP) attributable to the carbohydrate moieties and high content of hydroxy-amino acids, could have resulted in a lack of fixation in the gel by the staining solution. Finally, it is conceivable that the GMP is not stained by the techniques employed. Occasionally, faint patterns had been detected for the GMP in freshly stained gels and suggested that the latter effect was dominant. No conclusions were reached from these chance observations; however, heterogeneity in the GMP fraction appeared to be present.

It is rather surprising that the whole κ -casein, composed of one major and at least five minor components, yields primarily two para- κ -casein components. Similar results were obtained by Mackinlay and Wake (15). It is possible that the minor components of κ -casein represent varying compositions in the carbohydrate or peptide portions of the molecule, and with release of GMP, identical para- κ -caseins are obtained.

Polyacrylamide-gel electrophoresis in aluminum lactate at pH 4.0 did not result in as good a resolution as that obtained at pH 9.2. In Figure 2, it can be seen that the reduced para- κ -casein (Pattern 3) has a mobility approximately 60% greater than reduced κ -casein (Pattern 1), which can be attributed to the loss of the acidic GMP. Pattern 2 of the unreduced para- κ shows extensive smearing and streaking comparable to that observed with native κ -casein, which disappeared upon addition of mercaptoethanol and indicates a possible spectrum of molecular sizes arising from intermolecular disulfide bonding (15, 36).

Previous amino acid analyses of κ -casein were carried out on preparations from pooled milk (7, 8, 12, 28), in contrast to the analyses presented in Table 1, carried out on κ -casein derived from a single cow typed with respect to its major reduced κ -casein component. In addition, one reported analysis (8) was based on a single time of hydrolysis, whereas a second (12) was an average of three analyses made on three different preparations. In all cases, wherever it can be determined, the number of individual analyses performed was less than reported in the present paper and, in all cases except the present, suffer from a lack of any indicated

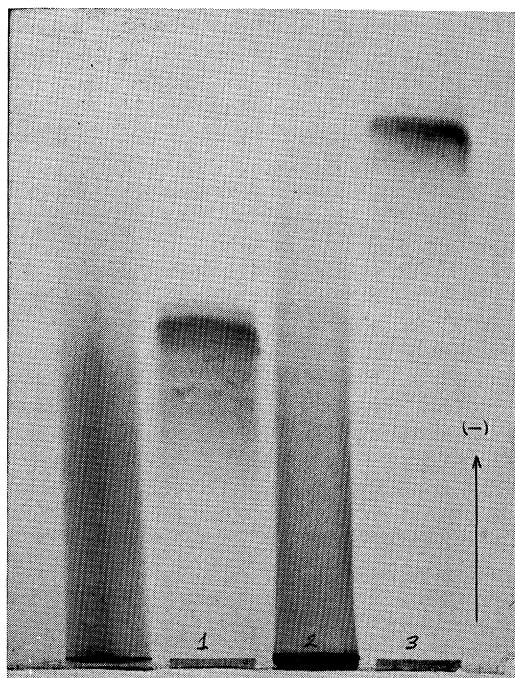


FIG. 2. Polyacrylamide-gel electrophoresis of κ -casein and para- κ -casein in aluminum lactate buffer, pH 4.0-4.5 M urea. Reduced κ -casein (1), para- κ -casein (2), and reduced para- κ -casein (3).

statistical reliability measure. The polypeptide portion of the present κ -casein preparation consists of 156-158 amino acid residues having a total molecular weight of 17,900, exclusive of tryptophan residues, reported to number 1 (12) or 2 (7). The number of residues of tryptophan per mole of protein (molecular weight 20,000) was 2.2 when calculated using the specific extinction coefficient of 12.2 (37) for κ -casein, molar extinction coefficients for cystine, tyrosine, and tryptophan given by Wetlaufer (33), and the values of 1 mole of cystine and 9 moles of tyrosine per mole of κ -casein reported in Table 1.

The single amino acid composition of para- κ -casein reported (11) suffers from the same deficiencies as noted above for κ -casein analyses. The data indicate that para- κ -casein from the present studies consists of 116-117 amino acid residues with a total molecular weight of 13,500, again exclusive of tryptophan residues reported to number 1-2 (11).

The amino acid composition of the peptidic portion of the GMP derived from κ -casein has been reported by several investigators (3, 8, 10, 19). In three instances (3, 8, 19) only a single time of hydrolysis was employed and in the remaining instance (10) two hydrolysis

times were employed. Again, no statistical data were presented and all preparations were derived from κ -caseins from pooled milk. The amino acid compositions, though similar in many respects, are not identical.

In the analysis of the two GMP's in the present case, small but significant amounts of aromatic and basic amino acids were found. These amino acids (phenylalanine and arginine) presumably are derived from contaminating peptidic material resulting from the tertiary phase of the rennin reaction (4). Blondel-Queroix and Alais (5) have reported at least four components in the supernatant after rennin action on κ -casein, one of which was thought to be a degradation product of the GMP. The data presented here indicate a GMP of 45-46 amino acids, which results in a total residue weight of 5,000. The virtual absence of absorption at 280 m μ would seem to rule out the presence of tryptophan. The calculated difference of amino acid residues between κ -casein and para- κ -casein values reported here is 42, resulting in a total residue weight of 4,400, slightly lower than the value determined above. Since the calculated residue numbers for the GMP are in fairly good agreement with the experimentally determined residue numbers, it is unlikely that the 12% TCA-insoluble fraction removed from the digest supernatant (see Materials and Methods) could materially affect the compositional data reported. The higher value of 45-46 for the GMP found experimentally can be accounted for by the higher residue numbers determined for glutamic acid, isoleucine, and possibly lysine. The most striking difference from other compositional data for GMP preparations is the finding of one residue of methionine/mole of GMP. This was true for both GMP preparations analyzed. This may be a consequence of the use of κ -casein from a single cow, with the genetic implications inherent in such a study. However, this must be verified by studying the rennin reaction with other preparations from individual cows. As noted above, Delfour et al. (6) have also recently reported the presence of methionine in GMP preparations. In addition, it should be pointed out that both reduced κ -casein and reduced para- κ -casein exhibit heterogeneity on gel-electrophoresis, even with preparations from individual animals. As previously mentioned, each of the components found in reduced κ -casein by this technique can act as a substrate for the rennin reaction. This is true even after the thiol groups are stabilized by alkylation (36) or sulfonation (15). Investigations are presently in progress to isolate the individual, reduced, and alkylated κ -ca-

sein components in pure form, to make it possible to study the rennin reaction in as well-defined a system as possible. The present data indicate that, as far as the peptide moieties involved in the rennin reaction go, there seems to be fairly good agreement in the summation of para- κ -casein plus GMP with total κ -casein. This again confirms κ -casein as the true site of rennin action and confirms and strengthens the hypothesis of Alais and Jollès (2) concerning the nature of the rennin reaction.

ACKNOWLEDGMENT

We thank Mr. J. N. Boyd, Biometrical Services, ARS, for calculating the measure of repeatability for the data in Table 1.

REFERENCES

- (1) ALAIS, C. 1956. Étude des Substances azotées nonprotéiques (NPN) séparées de la Caséine du Lait de Vache sous l'Action de la Présure. XIVth Intern. Dairy Congr. Proc. (Rome), 2 (Pt. 2), 823.
- (2) a. ALAIS, C. 1963. L'Action de la Présure sur la Caséine (2). Lait, 43: 481.
b. ALAIS, C., AND JOLLÈS, P. 1964. L'Action de la Présure sur la Caséine (1). Lait, 44: 138.
c. ALAIS, C., AND JOLLÈS, P. 1964. L'Action de la Présure sur la Caséine (Fin.). Lait, 44: 259.
- (3) ALAIS, C., BLONDEL-QUÉROIX, J., AND JOLLÈS, P. 1964. Étude des Substances Solubles Formées au Cours de la Réaction de la Présure sur la Caséine (II) Composition Chimique Fractionnement par l'Acide Trichloracétique. Bull. Soc. Chim. Biol., 46: 973.
- (4) ALAIS, C., MOCQUOT, G., NITSCHMANN, HS., AND ZÄHLER, P. 1953. Das Lab und seine Wirkung auf das Casein der Milch (VII). Über die Abspaltung von Nicht-Protein-Stickstoff (NPN) aus Casein durch Lab und ihre Beziehung zur Primärreaktion der Labgerinnung der Milch. Helv. Chim. Acta, 36: 1955.
- (5) BLONDEL-QUÉROIX, J., AND ALAIS, C. 1964. Étude des Substances Solubles Formées au Cours de la Réaction de la Présure sur la Caséine l'Étude Électrophorétique et Chromatographique. Bull. soc. chim. biol., 46: 963.
- (6) DELFOUR, A., JOLLÈS, J., ALAIS, C., AND JOLLÈS, P. 1965. Caseino-Glycopeptides: Characterization of a Methionine Residue and of the N-terminal Sequence. Biochem. Biophys. Research Commun., 19: 452.
- (7) HIPPEL, N. J., BASCH, J. J., AND GORDON, W. G. 1961. Amino Acid Composition of α_1 , α_2 , and α_3 -Caseins. Arch. Biochem. Biophys., 94: 35.
- (8) HUANG, F. Y-Y, HENNEBERRY, G. O., AND BAKER, B. E. 1964. Studies on Casein. (VII). The Carbohydrate Constitution of Glycopeptidic Material Isolated from Enzymic Hydrolysates of κ -Casein. Biochim. et Biophys. Acta, 83: 333.
- (9) JOLLÈS, P., AND ALAIS, C. 1960. Étude du Glycopeptide obtenu par Action de la Présure ("Rennin") sur la Caséine κ du Lait de Vache. Comp. rend. acad. sci., Paris, 251: 2605.
- (10) JOLLÈS, P., ALAIS, C., AND JOLLÈS, J. 1961. Étude Comparée des Caséino-Glycopeptides Formés par Action de la Présure sur les Caséines de Vache, de Brebis et de Chèvre. Biochim. et Biophys. Acta, 51: 309.
- (11) JOLLÈS, P., ALAIS, C., AND JOLLÈS, J. 1962. Amino Acid Composition of κ -Casein and Terminal Amino Acids of κ - and Para- κ -Casein. Arch. Biochem. Biophys., 98: 56.
- (12) JOLLÈS, P., ALAIS, C., AND JOLLÈS, J. 1963. Étude de la Caséine κ de Vache Caractérisation de la Liaison Sensible à l'Action de la Présure. Biochim. et Biophys. Acta, 69: 511.
- (13) JONES, R. W., AND CLUSKEY, J. E. 1963. Preparation of Aluminum Lactate. Cereal Chem., 40: 589.
- (14) a. LINDQVIST, B. 1963. Casein and the Action of Rennin—Part 1. Dairy Sci. Abstrs., 25: 257.
b. LINDQVIST, B. 1963. Casein and the Action of Rennin—Part 2. Dairy Sci. Abstrs., 25: 299.
- (15) MACKINLAY, A. G., AND WAKE, R. G. 1964. The Heterogeneity of κ -Casein. Biochim. et Biophys. Acta, 93: 378.
- (16) MATTENHEIMER, H., NITSCHMANN, HS., AND ZÄHLER, P. 1952. Das Lab und seine Wirkung auf das Casein der Milch. (VI). Über die Phosphatasewirkung des Labes. Helv. Chim. Acta, 35: 1970.
- (17) MCKENZIE, H. A., AND WAKE, R. G. 1961. An Improved Method for the Isolation of κ -Casein. Biochim. et Biophys. Acta, 47: 240.
- (18) NEELIN, J. M. 1964. Variants of κ -Casein Revealed by Improved Starch Gel Electrophoresis. J. Dairy Sci., 47: 506.
- (19) NITSCHMANN, HS., AND BEEBY, R. 1960. Rennin and Its Action on Milk Casein. (XIV). Amino Acid Composition of a Glycomacropeptide Released from κ -Casein by Rennin. Chimia, 14: 318.
- (20) NITSCHMANN, HS., AND HENZI, R. 1959. Das Lab und seine Wirkung auf das Casein der Milch. (XIII). Untersuchung der bei der Labung in Freiheit gesetzten Peptide. Helv. Chim. Acta, 42: 1985.
- (21) NITSCHMANN, HS., AND VARIN, R. 1951. Das Lab und seine Wirkung auf das Casein der Milch. (IV). Die Proteolyse des Caseins durch kristallisiertes Lab. Helv. Chim. Acta, 34: 1421.
- (22) NITSCHMANN, HS., WISSMANN, H., AND

- HENZI, R. 1957. Concerning a Glycomacropeptide, a Cleavage Product of Casein, Obtained by the Action of Rennet. *Chimia*, 11: 76.
- (23) NITSCHMANN, HS., AND ZAHLER, P. 1950. Das Lab und seine Wirkung auf das Casein der Milch. (III). Entstehen bei der Labgerinnung der Milch gerinnungsaktive Stoffe? *Helv. Chim. Acta*, 33: 854.
- (24) PETERSON, R. F. 1963. High Resolution of Milk Proteins Obtained by Gel Electrophoresis. *J. Dairy Sci.*, 46: 1136.
- (25) PIEZ, K. A., AND MORRIS, L. 1960. A Modified Procedure for the Automatic Analysis of Amino Acids. *Anal. Biochem.*, 1: 187.
- (26) SCHMIDT, D. G. 1964. Starch-Gel Electrophoresis of κ -Casein. *Biochim. et Biophys. Acta*, 90: 411.
- (27) SCHRAM, E., MOORE, S., AND BIGWOOD, E. J. 1954. Chromatographic Determination of Cystine as Cysteic Acid. *Biochem. J.*, 57: 33.
- (28) SWAISGOOD, H. E., BRUNNER, J. R., AND LILLEVIK, H. A. 1964. Physical Parameters of κ -Casein from Cow's Milk. *Biochem.*, 3: 1616.
- (29) TSUGO, T., AND YAMAUCHI, K. 1960. Specific Liberation of Nonprotein Nitrogen from κ -Casein Fraction by Rennin and Pepsin. *Bull. Agr. Chem. Soc., Japan*, 24: 96.
- (30) WAKE, R. G. 1949. Casein. V. The Action of Rennin on Casein. *Australian J. Biol. Sci.*, 12: 479.
- (31) WAKE, R. G. 1957. Action of Rennin on Casein. *Australian J. Sci.*, 20: 147.
- (32) WAUGH, D. F., AND VON HIPPEL, P. H. 1956. κ -Casein and the Stabilization of Casein Micelles. *J. Am. Chem. Soc.*, 78: 4576.
- (33) WETLAUFER, D. B. 1962. Ultraviolet Spectra of Proteins and Amino Acids. VII. Analytical Applications. *Advances in Protein Chem.*, 17: 375.
- (34) WOYCHIK, J. H. 1964. Polymorphism in κ -Casein of Cow's Milk. *Biochem. Biophys. Research Comm.*, 16: 267.
- (35) WOYCHIK, J. H. 1965. Phenotyping of κ -Caseins. *J. Dairy Sci.*, 48: 496.
- (36) WOYCHIK, J. H. 1965. Preparation and Properties of Reduced κ -Casein. *Arch. Biochem. Biophys.*, 109: 542.
- (37) ZITTLE, C. A., AND CUSTER, J. H. 1963. Purification and Some of the Properties of α_s -Casein and κ -Casein. *J. Dairy Sci.*, 46: 1183.